Table 1. ***Adult mouse cardiac myocyte isolation and culture procedure overview.***

Procedure	Description	Notes
Anesthesia	Isoflurane (3% induction/1.5% maintenance) with 100% O_2.	Isoflurane will reduce cardiac ischemia. Ketamine, and other similar anesthetics, have a slow onset and reduce respiration significantly, increasing the risk of ischemia.
Remove heart/cannulate	Identify and cut the aorta; try to leave about a 2mm section for cannulation.	Speed is the key to a successful cannulation. Also, proper cannula placement is essential; insertion too far into the left ventricle will not allow for adequate perfusion of the coronary arteries.
		The cannula is a 20-g needle, filed flat.
		Marking the cannula at 1 and 2 mm from the tip will help in positioning the heart.
		Arresting the heart with cold perfusion buffer was not beneficial.
Perfusion	Perfuse 4 min with Ca^{2+} free buffer.	Perfusion buffer is not oxygenated. Oxygenation had no beneficial effect on myocyte yield.
		Perfusion buffer and digestion buffer pH is maintained with Na HEPES rather than bubbling with 5% CO_2.
	Perfuse 8 to10 min with enzyme.	Enzyme: Blendzyme type 1 (Roche) with trypsin (Sigma), with 12.5 μM Ca^{2+}.
		Collagenase type II (Worthington) and a combination of Collagenase B and D (Roche) also worked well, but these enzymes can vary lot-to-lot. Yields were higher, but variability was increased.
	Constant flow: 3 ml/min.	Constant pressure (70 mm Hg) also works, but is harder to control.
		Lower flow rates do not work well.
Myocyte dissociation	Cut down heart, remove atria, and tease apart the ventricles with forceps.	When the heart is removed from the cannula, it should be pale and flaccid, and teasing apart the ventricle should be almost effortless.
	Inactivate the enzyme with serum.	BSA was initially used to inactivate the collagenase, but serum was better, and is now used.
	Finish digesting heart by pipetting through progressively smaller transfer pipettes.	If serum is added before the myocytes are dissociated completely, yields will be reduced.
		Inactivation buffer contains 12.5 μM Ca^{2+}.
Count myocytes	Determine the initial yield (see Table 2).	
Calcium reintroduction	Calcium is progressively increased from 12.5 μm to 1 mM.	Calcium reintroduction is done at room temperature in a 60-mm sterile bacterial dish (myocytes will not attach), so the myocytes can be visualized during reintroduction. Using a 15-ml tube will work, but there is increased chance of myocytes clumping together at the bottom of the tube. In addition, there was no advantage in preferential sedimentation of rod-shaped myocytes using the 15-ml tubes and gravity sedimentation of myocytes during calcium reintroduction.
	Finally, resuspend cells in plating medium: MEM w/Hanks' Balanced Salt Solution, with 5% fetal calf serum and 10 mM 2,3-	

	butanedione monoxime	
Count myocytes	Determine the number of rod-shaped myocytes for plating.	
Plating and attachment	Myocytes are plated: 50,000 rod-shaped myocytes/35-mm dish 150,000 rod-shaped myocytes/60-mm dish Myocytes are allowed to attach for 1 hr in a 37 °C incubator with 2% $C0_2$. MEM with Hanks' Balanced Salt Solution should be pH 6.9 to 7.0 in a 2% CO_2 incubator.	Dishes are coated with laminin. Other extracellular matrix components were tested, including fibronectin, collagen IV, and poly-L-lysine, but none were better than laminin.
Culture	After attachment, the medium is changed to Myocyte Culture Medium: MEM w/Hanks' Balanced Salt Solution, with 0.1 mg/ml BSA and 100 U/ml penicillin. MEM with Hanks' Balanced Salt Solution should be pH 6.9 to 7.0 in a 2% CO_2 incubator.	The slightly reduced pH of the culture medium (pH 6.9 to 7.0) helps the myocytes maintain their rod-shaped morphology up to 24 hr. Several agents were tested to try to improve rod-shaped morphology: 2, 3-butanedione monoxime (BDM)—contractile inhibitor Insulin, transferrin, and selenium (ITS) medium supplement Ascorbic acid (antioxidant) MnTMPyP (an SOD inhibitor) Caspase inhibitor (antiapoptotic) Bongkrekic acid (antiapoptotic) Verapimil (calcium channel antagonist) Low calcium medium Note: while BDM or ITS had only a minor effect on 24-hr maintenance of rod-shaped myocytes, they were essential for culture out to 72 hr (see Fig. 5).
Count myocytes	Count myocytes after attachment (1 hr); this is 0 hr (Table 3). Count myocytes after 24 hr (Table 3).	